Research Paper

Chronic depression of hypothalamic paraventricular neuronal activity produces sustained hypotension in hypertensive rats

Vera Geraldes[1], Nataniel Gonçalves-Rosa[1], Beihui Liu[2], Julian F. R. Paton[2] and Isabel Rocha[1,3]

[1]*Cardiovascular Autonomic Function Laboratory, Faculty of Medicine and Instituto de Medicina Molecular and* [3]*Centro de Cardiologia, University of Lisbon, Portugal*

[2]*School of Physiology & Pharmacology, Bristol Heart Institute, Medical Sciences Building, University of Bristol, Bristol BS8 1TD, UK*

New Findings

- **What is the central question of this study?**
  Will a chronic reduction of neuronal excitability within the paraventricular nucleus of the hypothalamus reduce arterial blood pressure and sympathetic activity in the long term in an animal model of neurogenic hypertension?
- **What is the main finding and its importance?**
  We show, for the first time, that overexpression of an inwardly rectifying potassium channel in the paraventricular nucleus provided a long-term (>60 days) antihypertensive response in conscious spontaneously hypertensive rats that was associated with a reduction in neurohumorally mediated vasoconstriction, enhanced baroreflex sensitivity and reduced peripheral chemosensitivity; no such response was observed in normotensive rats. Our results support the paraventricular nucleus as a therapeutic target for the chronic control of blood pressure in neurogenic hypertension.

Changes in the sympathetic nervous system are responsible for the initiation, development and maintenance of hypertension. An important central sympathoexcitatory region is the paraventricular nucleus (PVN) of the hypothalamus, which may become more active in hypertensive conditions, as shown in acute studies previously. Our objective was to depress PVN neuronal activity chronically by the overexpression of an inwardly rectifying potassium channel (hKir2.1), while evaluating the consequences on blood pressure (BP) and its reflex regulation. In spontaneously hypertensive rats (SHRs) and Wistar rats (WKY) lentiviral vectors (LVV-hKir2.1; LV-TREtight-Kir-cIRES-GFP5 4×10^9 IU and LV-Syn-Eff-G4BS-Syn-Tetoff 6.2×10^9 IU in a ratio 1:4) were stereotaxically microinjected bilaterally into the PVN. Sham-treated SHRs and WKY received bilateral PVN microinjections of LVV-eGFP (LV-Syn-Eff-G4BS-Syn-Tetoff 6.2×10^9 IU and LV-TREtight-GFP 5.7×10^9 IU in a ratio 1:4). Blood pressure was monitored continuously by radio-telemetry and evaluated over 75 days. Baroreflex gain was evaluated using phenylephrine (25 μg ml^{-1}, I.V.), whereas lobeline (25 μg ml^{-1}, I.V.) was used to stimulate peripheral chemoreceptors. In SHRs but not normotensive WKY rats, LVV-hKir2.1 expression in the PVN produced time-dependent and significant decreases in systolic (from 158 ± 3 to 132 ± 6 mmHg; $P < 0.05$) and diastolic BP (from 135 ± 4 to 113 ± 5 mmHg; $P < 0.05$). The systolic BP low-frequency band was reduced (from 0.79 ± 0.13 to 0.42 ± 0.09 mmHg^2; $P < 0.05$), suggesting reduced sympathetic vasomotor tone. Baroreflex gain was increased and peripheral chemoreflex depressed after PVN microinjection of LVV-hKir2.1. We conclude that the PVN plays a major role in long-term control of BP and sympathetic nervous system activity in SHRs.

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Experimental Physiology

This is associated with reductions in both peripheral chemosensitivity and respiratory-induced sympathetic modulation and an improvement in baroreflex sensitivity. Our results support the PVN as a powerful site to control BP in neurogenic hypertension.

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Corresponding author I. Rocha: Instituto de Fisiologia, Faculdade de Medicina de Lisboa, Av Prof Egas Moniz, 1649-028 Lisbon, Portugal. Email: isabelrocha@fm.ul.pt

Introduction

Essential arterial hypertension has now reached pandemic proportions, with an estimated one billion sufferers worldwide. The pathogenesis of essential arterial hypertension is multifactorial and not completely understood, but there is clear evidence that chronic elevation of sympathetic nervous system activity is a major contributor to the onset, development and maintenance of the hypertensive state (Grassi, 2004*b*; Guyenet, 2006; Fisher & Paton, 2012). In fact, the increase of sympathetic outflow to the heart results in increased cardiac output and neurally mediated vasoconstriction, leading to elevated blood pressure values (Schlaich *et al.* 2012). In 'white coat' and borderline hypertensive patients, sympathetic nerve activity to the arterioles supplying skeletal muscle is raised in comparison to healthy individuals (Grassi, 2004*a*; Smith *et al.* 2004). Excessive sympathetic activity may contribute to hypertrophy of vascular smooth muscle and cardiac muscle, brain hypoperfusion and inflammation, and becomes a major target to control in neurogenic hypertension (Zubcevic *et al.* 2011).

The evaluation of sympathetic activity can be achieved indirectly by applying mathematical tools such fast Fourier transform to blood pressure signals (Task Force of the European Society of Cardiology and the North American Society of Pacing and Electrophysiology, 1996). A power spectrum is generated, where the low frequencies (LFs) represent predominantly sympathetic activity and high frequencies (HFs) are related to parasympathetic tonus and respiration (Radaelli *et al.* 1994; Task Force of the European Society of Cardiology and the North American Society of Pacing and Electrophysiology, 1996; Furlan *et al.* 2000). These mathematical results have been addressed in several studies, which have suggested that sympathetic activity is a critical determinant of blood pressure fluctuations in a frequency range which is slower than the rate of respiration (Japundzic *et al.* 1990; Cerutti *et al.* 1991; Malliani *et al.* 1991).

Located in the hypothalamus, the paraventricular nucleus (PVN) is a major sympathoexcitatory area that becomes more active in conditions of hypertension, such as in the spontaneously hypertensive rat (SHR) model (Allen, 2002). Some authors have referred to this region as a command nucleus providing feedforward excitatory synaptic drives to co-ordinate lower brainstem cardiovascular and respiratory motor activity (Dampney *et al.* 2005). Activation of the PVN promotes an increase in sympathetic output and a pressor effect mediated via direct and indirect projections (via the rostral ventrolateral medulla) to the spinal cord (Caverson *et al.* 1984; Shafton *et al.* 1998; Pyner & Coote, 2000; Hardy, 2001).

Both electrical stimulation and chemical manipulation of PVN neurons with bicuculline (a $GABA_A$ receptor antagonist) or glutamate elevated sympathetic nerve activity and caused hypertension in anaesthetized and conscious rats (Kannan *et al.* 1989; Zhang *et al.* 2002). In contrast, acute inhibition of the PVN with GABA or muscimol reduces the blood pressure and sympathetic nerve activity in SHRs (Allen, 2002). Lesions of the PVN or transection of the brain caudal to the hypothalamus promotes a decrease in blood pressure in SHRs but not in Wistar–Kyoto (WKY) rats (Yamori & Okamoto, 1969; Goto *et al.* 1981; Ciriello *et al.* 1984; Herzig *et al.* 1991; Takeda *et al.* 1991).

Long-term manipulation of neurone excitability can be performed by expressing a human inwardly rectifying potassium channel (hKir2.1) under the control of a selective neuronal promoter, such as synapsin (Duale *et al.* 2005, 2007). Inwardly rectifying potassium channels, such as Kir2.1, are endogenously expressed in rat brain and have recently been overexpressed as a means to reduce neuronal membrane excitability (Yu *et al.* 2004; Duale *et al.* 2007; Mizuno *et al.* 2007; Okada & Matsuda, 2008; Yoon *et al.* 2008; Howorth *et al.* 2009). Their long-term expression can be achieved by the use of lentiviral vectors (LVVs) derived from human immunodeficiency virus (Coleman *et al.* 2003). Therefore, using a LVV to overexpress hKir2.1 channels within the PVN, we sought to determine the long-term influence of this nucleus on the control of blood pressure, heart rate, sympathetic activity and respiration in SHRs, as well as homeostatic reflex control mechanisms.

Methods

All the experimental procedures were in accordance with the European and Portuguese Law on animal welfare and had the approval of the ethics committee of the Faculty of Medicine, University of Lisbon, Portugal. Male Wistar–Kyoto rats ($n = 15$) and SHRs ($n = 15$) were used, aged 12 weeks and weighing 363 ± 8 g. Animals, synchronized to a 12 h–12 h light–dark cycle (light on at 07.00 h and light off at 19.00 h), were housed individually and allowed to freely move in standard plastic cages. Food and water were available *ad libitum*.

Viral vector construction and validation

Lentiviral vector construction was based on previous studies (Waki *et al.* 2003; Duale *et al.* 2007). Briefly, LVV-eGFP, used for the sham-treated group, was a mix of LV-TREtight-GFP 5.7×10^9 IU and LV-Syn-Eff-G4BS-Syn-Tetoff 6.2×10^9 IU in a ratio 1:4. This binary system expresses enhanced green fluorescent protein (eGFP). The LVV-hKir2.1 is a mix of LV-TREtight-Kir-cIRES-GFP 5.4×10^9 IU and LV-Syn-Eff-G4BS-Syn-Tetoff 6.2×10^9 IU in a ratio 1:4, which expresses eGFP and expresses human inwardly rectifying potassium channels (hKir2.1) in neurones. Validation of transduction efficacy and transgene expression was assessed as described previously by Duale *et al.* (2007) and included mRNA expression, immunocytochemical and electrophysiological data.

Microinjection sites

Initially, we fine tuned our stereotaxic co-ordinates for bilateral PVN microinjections in five SHRs and five WKY rats anaesthetized with sodium pentobarbitone (60 mg kg^{-1}, I.P., Hikma Pharmaceuticals, London, UK). Bilateral microinjections (0.05 μl) of LVV-eGFP were performed. Using fluorescence microscopy and histological reconstruction, we determined the correct co-ordinates for PVN injections and the amount of LVV-eGFP needed to limit transduction to the confines of the PVN.

Surgery

Spontaneoulsy hypertensive rats were divided into two groups according to the microinjection content, i.e. LVV-hKir2.1 ($n = 8$) and LVV-eGFP ($n = 7$). A control group of WKY rats, with matching age, sex and number of individuals, underwent the same surgical and experimental protocol.

Implantation of telemetry probes. Rats were implanted with radio-telemetry probes (DSI, St. Paul, Minnesota, MN, USA) in the abdominal aorta under general anaesthesia (sodium pentobarbitone, 60 mg kg^{-1}, I.P., Hikma Pharmaceuticals). Animals were allowed to recover for 15 days. Similar anaesthetic and surgical protocols were applied to WKY rats ($n = 15$).

Bilateral microinjection in the PVN. Two weeks after the probes were implanted, SHRs ($n = 8$) and WKY rats ($n = 8$) under general anaesthesia (sodium pentobarbitone, 60 mg kg^{-1}, I.P., Hikma Pharmaceuticals) were placed in a stereotactic frame (Kopf Instruments, Tujunga, CA, USA), and a craniotomy was performed using our previously determined co-ordinates for LVV-hKir2.1 microinjections (0.05 μl) into the PVN (Bregma, −1.6 mm; Lateral, ±1.41 mm; Deep, 7.4 mm; pipette angle, 10 deg to bregma; Paxinos & Watson, 1986). Sham-treated rats were microinjected in the same region with LVV-eGFP (SHRs, $n = 7$; and WKY rats, $n = 7$). All microinjections were performed bilaterally. Animals of all groups were allowed to recover and monitored by telemetry for 60 days. Heart rate (HR) and blood pressure [BP; systolic (SBP), diastolic and mean] were recorded continuously.

Metabolic evaluation

Rats were housed for 24 h in metabolic cages to evaluate body weight, intake of food and fluid and production of urine and faeces. Measurements were performed before and 59 days after each microinjection.

Cardiorespiratory reflex evaluation

At 60 days, animals were anaesthetized (sodium pentobarbitone, 60 mg kg^{-1}, I.P., Hikma Pharmaceuticals). The trachea was cannulated below the larynx to record tracheal pressure. The femoral and carotid arteries and femoral vein were cannulated. Rectal temperature was maintained at 38 ± 1°C by a servo-controlled heating blanket. The ECG was recorded with the use of needle electrodes inserted into the limbs, and HR was derived from the ECG. Baroreceptor and peripheral chemoreceptor reflexes were activated twice, with an interval of 5 min between stimuli. Baroreceptor reflex was stimulated by phenylephrine (0.2 ml, 25 μg ml^{-1} I.V.; Sigma Aldrich). Peripheral chemoreceptor reflex was stimulated with lobeline (0.2 ml, 25 μg ml^{-1}, Sigma Aldrich) injected retrogradely into the bifurcation of the common carotid artery. Heart rate, BP (systolic, diastolic and mean) and respiratory rate (RespR) were recorded continuously thought the experiment.

Histology and immunochemistry

Animals were terminally anaesthetized and immediately perfused transcardially with PBS (0.1 M; pH 7.4) followed by 4% paraformaldehyde (0.1 M; pH 7.4). The brain was removed and placed for 48 h in 15% (w/v) sucrose solution. Coronal sections (18 μm thick) were cut on a microtome and mounted on slides. The pipette tip location and the microinjection diffusion in the PVN were examined and documented. The microinjected contents (LVV-hKir2.1 or LVV-eGFP) containing eGFP allowed an estimation of virus dispersion. The eGFP-labelled fluorescent regions were identified using an epifluorescence microscope and plotted on standardized sections from the atlas of Paxinos & Watson (1986).

Western blot analysis

The expression of hKir2.1 in the PVN was analysed by Western blot 60 days after the microinjection of

LVV-hKir2.1 ($n = 8$) or LVV-eGFP in SHRs ($n = 7$). The PVN was dissected from both groups and homogenized by sonication in ice-cold RIPA buffer (Sigma, St. Louis, MO, USA) supplemented with a cocktail of protease inhibitors (complete mini; Roche). Proteins were extracted from the homogenates by centrifugation at 5000*g* for 10 min at 4°C, and protein concentration was determined with a Bio-Rad DC Protein Assay kit. Proteins were resolved by electrophoresis on a 10% Tris–glycine SDS-PAGE gel and transferred to a polyvinylidene fluoride membrane (Millipore, Bedford, MA, USA). Membranes were blocked with 5% milk in Tween/Tris-buffered saline and incubated overnight at 4°C with rabbit anti-hKir2.1 polyclonal antibody (Abcam, Cambridge, UK). After washing, membranes were incubated for 1 h at room temperature with HRP-conjugated goat anti-rabbit antibody (Bio-Rad, Hercules, CA, USA), and immunoreactive proteins were detected by Immobilon Western Chemiluminescent HRP Substrate (Millipore, Bedford, MA, USA) and visualized using Curix 60 (AGFA, Greenville, SC, USA). Membranes were stripped with 0.1 M glycine (pH 2.2) and reprobed with the α-tubulin antibody (Santa Cruz Biotechnology, Dallas, TX, USA) for loading control.

Data acquisition and analysis

Telemetric data were acquired at 1 kHz and analysed with suitable software (LabChart6, Powerlab; ADInstruments, Oxford, UK). Mean values of HR, BP (systolic, diastolic and mean) and RespR were extracted.

Baroreceptor and chemoreceptor reflex. The baroreceptor reflex gain (BRG) was quantified by calculating ΔHR/ΔBP (in beats per minute per millimetre of mercury). Chemoreceptor (ChR) reflex was calculated through the RespR derived from the tracheal pressure before and after stimulation with lobeline, as follows: $\Delta\text{ChR} = \text{RespR}_{\text{lobeline}} - \text{RespR}_{\text{basal}}$. Blood pressure and HR were also evaluated.

Analysis of BP and HR variability. Systolic BP and R–R interval data were analysed (period of 3 min) in the frequency domain (Fast Fourier Transform), using the in-house software Fisiosinal (Tavares, 2011), to evaluate sympathetic (LF band, 0.15–0.6 Hz of systolic BP) and parasympathetic activity (HF band, 0.6–2.0 Hz of HR) over time (Task Force of the European Society of Cardiology and the North American Society of Pacing and Electrophysiology, 1996; Marques-Neves *et al.* 2004).

Circadian light/dark heart rate and blood pressure profile. Mean BP and HR values were calculated using the continuous telemetric data and compared between light (07.00–19.00 h) and dark phases (19.00–07.00 h).

Statistical analysis

Comparisons were performed between groups for the same period and within the same group, before and after the microinjections. For the statistical analysis, Student's paired *t* test and ANOVA for comparisons between groups were used. All data were expressed as means ± SEM and passed the normality test. Significance was taken as $P < 0.05$.

Results

Effect of microinjection of LVV-hKir2.1 or LVV-eGFP on 24 h mean values of blood pressure, heart rate and respiration

Basal BP values (recorded before microinjections) in conscious SHRs ($n = 15$) were 158 ± 3 mmHg for systolic BP, 135 ± 4 mmHg for diastolic BP and 142 ± 3 mmHg for mean BP, and were significantly higher than the values for WKY rats (119 ± 3, 91 ± 2 and 101 ± 1 mmHg, respectively; $n = 15$; $P < 0.0001$). The SHRs showed a higher baseline respiratory rate than WKY rats (77 ± 5 *versus* 61 ± 4 breaths min^{-1}, respectively; $P < 0.05$) as well as a lower HR (311 ± 5 and 367 ± 9 beats min^{-1}; $P < 0.0001$).

Thirty days after LVV-hKir2.1 microinjection, a significant BP decrease ($P < 0.05$) was first observed, but in order to evaluate its persistence, animals were monitored for a further 30 days. On the 60th day after lentiviral microinjection, values for SHRs for systolic, diastolic and mean BP were 132 ± 6, 113 ± 5 and 120 ± 5 mmHg, respectively, corresponding to a decrease in pressure of 26, 22 and 22 mmHg, respectively ($P < 0.01$; Fig. 1). These BP changes were accompanied by a lowering of HR (295 ± 3 beats min^{-1}, $P = 0.099$) but RespR remained unchanged. The decreased BP and HR values approached those of normotensive animals. At the same time, SHRs microinjected with LVV-eGFP were showing increased values of systolic (174 ± 10 mmHg; $P > 0.05$), diastolic (149 ± 11 mmHg; $P > 0.05$) and mean BP (157 ± 10 mmHg; $P > 0.05$), together with a significantly decreased HR (285 ± 6 beats min^{-1}; $P < 0.01$). This profile of BP and HR changes was expected and is a consequence of maturation. In contrast, no significant changes in BP, HR and RespR were observed in WKY rats during the 60 day duration of the experimental protocol.

Effect of LVV-hKir2.1 microinjection on sympathetic output measured indirectly

Spontaneously hypertensive rats showed putative evidence for an overall decrease of cardiovascular autonomic outflow at 60 days after LVV-hKir2.1 microinjection when compared with basal autonomic output at day 0. In fact,

by using fast Fourier transform applied to SBP and R–R intervals, a decrease of LF_{SBP}/HF_{RR} ratio (from 0.07 ± 0.02 to 0.04 ± 0.01 mmHg^2 ms^{-2}; $P > 0.05$) was observed, mainly due to a strong decrease in sympathetic output expressed by LF_{SBP} band power (from 0.79 ± 0.13 to 0.42 ± 0.09 mmHg^2; $P < 0.05$). In SHRs, the basal HF_{SBP} (0.75 ± 0.10 mmHg^2) was first reduced at 40 days and persisted until 60 days (0.33 ± 0.10 mmHg^2; $P < 0.05$) after LVV-hKir2.1, but it was unchanged in the LVV-eGFP group (0.82 ± 0.38 mmHg^2). Interestingly, LF SBP was significantly reduced by 20 days after LVV-hKir2.1 microinjection and occurred before the fall in SBP. In contrast, at 60 days the LF_{SBP}/HF_{RR} ratio for SHR LVV-eGFP was 0.08 ± 0.03 mmHg^2 ms^{-2} and the LF was 0.86 ± 0.21 mmHg^2 ($P > 0.05$). The variations of mean LF_{SBP} and LF_{SBP}/HF_{RR}, at 10 day intervals for each SHR group, are depicted in Fig. 2. For WKY rats in basal conditions, the LF_{SBP} and LF_{SBP}/HF_{RR} ratio were 3.23 ± 0.36 mmHg^2 and 0.43 ± 0.14 mmHg^2 ms^{-2}, respectively. No significant changes in LF and LF_{SBP}/HF_{RR} ratio were observed for WKY LVV-hKir2.1 (3.11 ± 0.44 mmHg^2 and 0.40 ± 0.23 mmHg^2 ms^{-2}, respectively) and WKY LVV-eGFP rats (2.56 ± 0.48 mmHg^2 and 0.22 ± 0.08 mmHg^2 ms^{-2}, respectively).

Arterial baroreflex gain and peripheral chemoreflex responsiveness

The injection of phenylephrine triggered, in all animal groups, a progressive increase in mean BP, which was accompanied by a progressive reduction in HR. In SHRs, BRG increased significantly after LVV-hKir2.1 microinjection and approached the values of the normal control rats. The SHR LVV-hKir2.1 group had a higher BRG than the SHR LVV-eGFP group (0.51 ± 0.06 *versus* 0.33 ± 0.03 beats min^{-1} mmHg^{-1}, respectively; $P < 0.05$; Fig. 3). Interestingly, the BRG of WKY LVV-hKir2.1 rats (1.29 ± 0.18 beats min^{-1} mmHg^{-1}) was also increased in comparison to the WKY LVV-eGFP group (0.41 ± 0.02 beats min^{-1} mmHg^{-1}; $P < 0.0001$), despite all cardiovascular variables remaining unchanged.

Respiratory rate remained unchanged throughout the full experimental protocol in all animal groups, before and after the lentiviral microinjection. At 60 days after microinjection, the baseline values of respiratory rate in the anaesthetized animals were 76 ± 3.4, 81 ± 4.9, 80 ± 4.5 and 67 ± 3.5 breaths min^{-1}, respectively, for SHR and WKY LVV-hKir2.1, SHR and WKY LVV-eGFP. However, peripheral chemoreceptor reflex activation with lobeline elicited a hyperventilatory reflex response of different magnitude according to the animal group. The SHR LVV-hKir2.1 animals showed a decreased ventilatory response when compared with the SHR LVV-eGFP group ($\Delta 24.4 \pm 3.4$ *versus* $\Delta 38.1 \pm 4.9$ breaths min^{-1}, respectively; $P < 0.05$; Fig. 3). In contrast, there were no differences in the ventilatory response between WKY LVV-hKir2.1 and WKY-eGFP groups ($\Delta 23.3 \pm 5.9$ breaths min^{-1} for WKY LVV-hKir2.1 and $\Delta 24.8 \pm 4.2$ breaths min^{-1} for WKY LVV-eGFP). Mean BP responses to chemoreflex activation in SHR LVV-hKir2.1 animals (from 140 ± 7 to 154 ± 9 mmHg) were depressed compared with SHR LVV-eGFP rats (179 ± 9 to 193 ± 9 mmHg; $P < 0.05$), but HR responses were not different (from 337 ± 23 to 359 ± 12 *versus* from 373 ± 10 to 362 ± 13 beats min^{-1}, respectively). For the two WKY groups, changes in BP and HR in response to peripheral chemoreflex activation were not different.

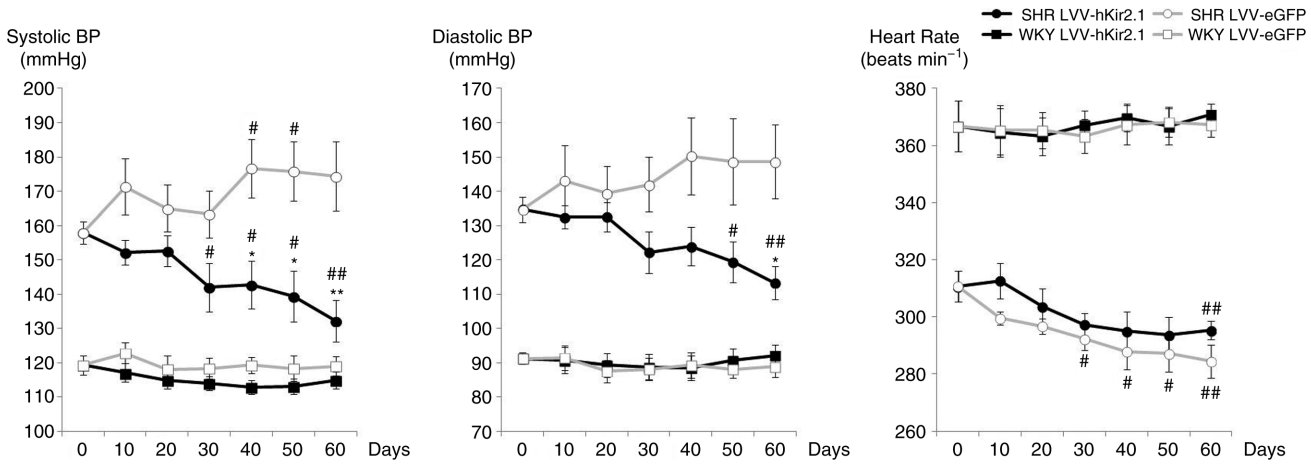

Figure 1. Effect on systolic blood pressure, diastolic blood pressure and heart rate before (0 days) and after microinjection of LVV-hKir2.1 ($n = 7$) or LVV-eGFP ($n = 7$)
$^*P < 0.05$, $^{**}P < 0.01$, statistically significant differences between spontaneously hypertensive rat (SHR) LVV-hKir2.1 and SHR LVV-eGFP groups. #$P < 0.05$, ##$P < 0.01$, statistically significant differences within the group.

Circadian variation of BP and HR and patterns of nocturnal blood pressure profile

In basal conditions and without any intervention, the pattern of circadian variation of BP and HR followed a similar trend, with lower BP values during the light phase relative to the dark phase. During the light phase, the systolic, diastolic and mean BP of SHRs were significantly higher than those for WKY rats (Table 1; $P < 0.0001$) over the same time period. The same type of variation was found for the dark phase, during which SHRs showed higher values for BP parameters than WKY rats ($P < 0.0001$; Table 1). Mean basal HR followed these variations in BP inversely. The HR was significantly lower during the light and dark phases for SHRs than for WKY rats ($P < 0.01$; Table 1).

At 60 days after the LVV-hKir2.1 microinjection, SHRs showed a significant decrease of systolic, diastolic and mean BP during both the light and the dark phase (both $P < 0.01$; Table 1). A significant decrease of HR was observed during the light but not during the dark phase ($P > 0.05$). For the SHR LVV-eGFP rats, HR, diastolic, systolic and mean BP values for the light phase and dark phase were increased as expected at 60 days (Table 1). Finally, in WKY LVV-hKir2.1 as well as WKY LVV-eGFP rats there was an increase in BP during the dark phase without a distinct circadian rhythm. This profile was maintained at 60 days after LVV-hKir2.1 and LVV-eGFP PVN microinjections (Table 1).

Metabolic evaluation

A significant decrease in food intake was observed in the SHR LVV-hKir2.1 group at 60 days after the microinjection (Table 2). No other significant changes were found in body weight, water intake, faeces and urine production for all groups, before and after the microinjections, suggesting that the physical inactivity due to social isolation (only one animal per cage) could have an impact on food consumption. Furthermore, animals were not subjected to an adaptation period to the metabolic cages, which could impact on our metabolic data, constituting a study limitation.

Histological, immunohistochemical and Western blot analysis

The microinjection sites were located within the PVN according to the rat atlas of Paxinos & Watson (1986). Enhanced green fluorescent protein was detected by fluorescence microscopy as fluorescence confined to a surface of 0.10–0.20 mm around the injection site. The eGFP did not penetrate the third ventricular ependymal lining. Through immunohistochemical studies, it was confirmed that PVN neurones expressed eGFP (Fig. 4).

The overexpression of hKir2.1 in the PVN was analysed using Western blot. The PVN dissected from SHRs microinjected with LVV-hKir2.1 showed an increased expression of hKir2.1, on average about ninefold increased when compared with the LVV-eGFP group (Fig. 4).

Discussion

In the present study, we investigated the effect of overexpressing an inwardly rectifying potassium channel

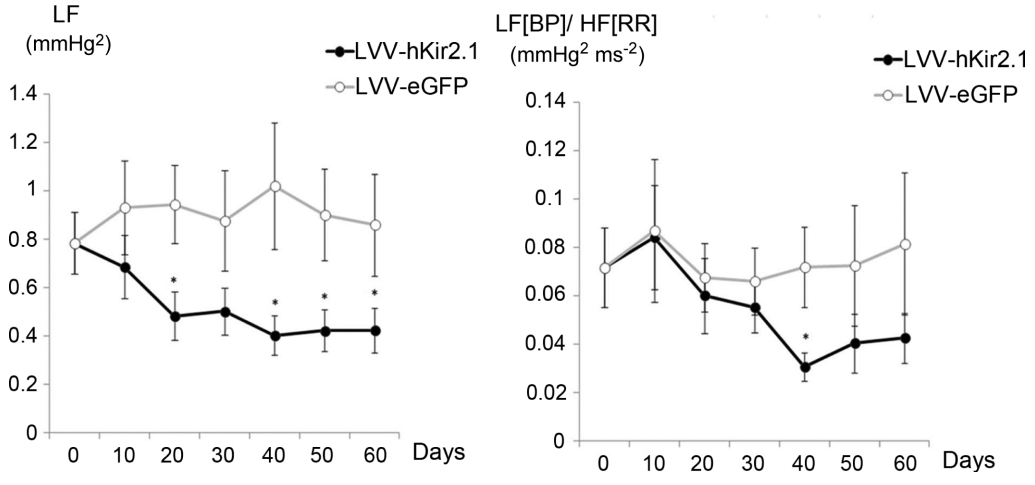

Figure 2. Mean (±SEM) low frequency (LF) and ratio of low (blood pressure) to high frequency (R–R interval) [LF(BP)/HF(RR)] before (0 days) and at 10 day intervals after the microinjection of LVV-hKir2.1 or LVV-eGFP in SHRs
Note that the fall in LF systolic blood pressure (SBP) occurred a week before the fall in SBP, suggesting a causative association. $^*P < 0.05$, statistically significant difference between groups.

in the PVN to lower neuronal activity, while measuring BP chronically, as well as its reflex control, in a rat model of hypertension. Our study is the first to demonstrate that chronic suppression of PVN neuronal activity in freely moving SHRs causes a sustained reduction in arterial blood pressure (>60 days) together with a decrease of sympathetic activity, a downregulation of peripheral chemoreflex responsiveness and an improvement of baroreflex gain. No such changes were found in the control groups of both rat strains that underwent comparable experimental protocols.

The PVN of the hypothalamus is well known for its importance in autonomic control and, in particular, for cardiovascular regulation. Several anatomical and electrophysiological studies have shown that PVN neurones project either directly to the spinal cord or to the rostral ventrolateral medulla (Coote, 2007), thereby accessing sympathetic neurones to modulate blood pressure (Hosoya *et al.* 1991; Loewy, 1991; Coote, 1995, 2005; Ranson *et al.* 1998; Motawei *et al.* 1999; Pyner & Coote, 1999, 2000; Badoer, 2001). As an example, electrolytic lesions of the PVN in SHRs elicited an acute reduction of sympathetic activity together with a decrease of blood pressure (Takeda *et al.* 1991). Other acute studies, performed under general anaesthesia, showed that muscimol injections into the PVN lowered BP and renal sympathetic nerve activity in both SHRs and WKY rats, indicating that this region was tonically active in both animal strains to control BP and peripheral sympathetic activity (Allen, 2002). In the SHR, sympathetic activity is known to be overactivated even before hypertension develops (Simms *et al.* 2009). Several studies have pointed out that the persistent increase in sympathetic tone is a major contributor to both the initiation and the maintenance of the hypertensive condition (Yamada *et al.* 1988; Grassi, 2004*b*; Smith *et al.* 2004; Guyenet, 2006; Fisher & Paton, 2012). In fact, increased sympathetic activity has been detected in normotensive individuals with a family history of hypertension and in individuals with essential hypertension, but not in those with secondary hypertension (Yamada *et al.* 1988; Grassi *et al.* 1998; Grassi, 2004*a*, 2009). Likewise, high plasmatic noradrenaline levels have also been associated with essential hypertension, being consistently increased in younger hypertensive patients (Grassi, 1998), and increased peripheral sympathetic nervous activity has been detected by microneurographic techniques in hypertensive patients (Anderson *et al.* 1989; Grassi, 1998; Greenwood *et al.* 1999; Mano, 2012). Several studies, both in human subjects and in animal models, have demonstrated an association between the circadian variation of BP values, the hypertensive condition, the sympathetic activation, the end-organ damage and the worsening of cardiovascular outcome (White, 2000; Pickering & Kario, 2001; Weber, 2002). Thus, the idea of a long-term modulation of the level of sympathetic activity, at its central origin, as a way to control and treat high blood pressure and to increase cardiovascular compliance, is very appealing. In particular, the manipulation of sympathetic cell excitability by modulation of K^+ channel expression, to hyperpolarize neuronal resting membrane potential, is an attractive hypothetical therapeutic strategy (Duale *et al.* 2007).

In the present work, our purpose was to depress the activity of PVN neurones chronically by the overexpression of K^+ channels exclusively in PVN

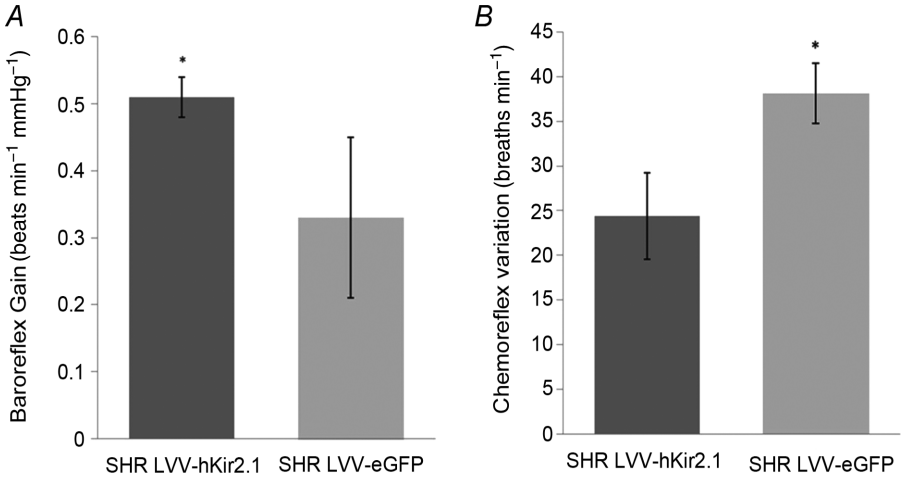

Figure 3. Effect of bilateral microinjections of LVV-hKir2.1 or LVV-eGFP into the paraventricular nucleus on baroreflex gain (*A*) and chemoreflex variation (*B*), 60 days after microinjection
In the SHR LVV-hKir2.1 group, there is an increase in the baroreflex gain and a decrease in the chemoreflex ventilatory response. $^*P < 0.05$, statistically significant differences between groups.

Table 1. Blood pressure (in millimetres of mercury) and heart rate (in beats per minute) during the light and dark phases for all groups before and 59 days after the microinjection

	Light phase				Dark phase			
Group	SBP	DBP	MBP	HR	SBP	DBP	MBP	HR
Basal								
SHR	156 ± 3	132 ± 3	140 ± 3	297 ± 6	160 ± 4	137 ± 4	145 ± 4	325 ± 6
WKY	118 ± 3	90 ± 2	100 ± 1	362 ± 9	120 ± 3	92 ± 2	102 ± 2	373 ± 11
59 days after microinjection								
SHR LVV-hKir2.1	131 ± 5*	113 ± 4*	119 ± 4*	271 ± 2	133 ± 7*	114 ± 6*	120 ± 6*	320 ± 5*
SHR LVV-eGFP	172 ± 11	145 ± 11	154 ± 10	264 ± 5	177 ± 10	152 ± 11	160 ± 10	305 ± 7
WKY LVV-hKir2.1	117 ± 4	87 ± 3	97 ± 2	340 ± 10	121 ± 2	91 ± 4	101 ± 3	378 ± 12
WKY LVV-eGFP	114 ± 2	88 ± 3	96 ± 2	354 ± 5	116 ± 2	92 ± 3	100 ± 2	389 ± 2

Values are expressed as means ± SEM. Abbreviations: DBP, diastolic blood pressure; HR, heart rate; MBP, mean blood pressure; and SBP, systolic blood pressure; SHR LVV-hKir2.1, Spontaneously hypertensive rats microinjected with LVV-hKir2.1; SHR LVV-eGFP, Spontaneously hypertensive rats microinjected with LVV-eGFP; WKY LVV-hKir2.1, Wistar Kyoto rats microinjected with LVV-hKir2.1; WKY LVV-eGFP, Wistar Kyoto rats microinjected with LVV-eGFP. $^*P < 0.01$, statistically significant difference between basal and day 59 values.

Table 2. Metabolic evaluation of spontaneously hypertensive rats before injection and 59 days afterwards

Group	ΔWeight (g)	Food (g)	Water (ml)	Faeces (g)	Urine (ml)
Before microinjection; basal conditions					
SHR LVV-eGFP	−1 ± 2.0	19 ± 3.5	27 ± 2.2	9 ± 2.1	11 ± 1
SHR LVV-hkir2.1	−1 ± 1.4	24 ± 1.3	40 ± 4.2	14 ± 3.1	16 ± 3.5
After microinjection (at 59 days)					
SHR LVV-eGFP	−3 ± 1.3	27 ± 1.6	31 ± 4.0	13 ± 1.5	12 ± 0.9
SHR LVV-hkir2.1	−1 ± 0.6	20 ± 0.6*	32 ± 4.9	8 ± 0.9	16 ± 2.1

Values are expressed as means ± SEM. Abbreviations: SHR LVV-hKir2.1, Spontaneously hypertensive rats microinjected with LVV-hKir2.1; SHR LVV-eGFP, Spontaneously hypertensive rats microinjected with LVV-eGFP; WKY LVV-hKir2.1, Wistar Kyoto rats microinjected with LVV-hKir2.1; WKY LVV-eGFP, Wistar Kyoto rats microinjected with LVV-eGFP. $^*P < 0.05$, statistically significant difference between basal and day 59 values.

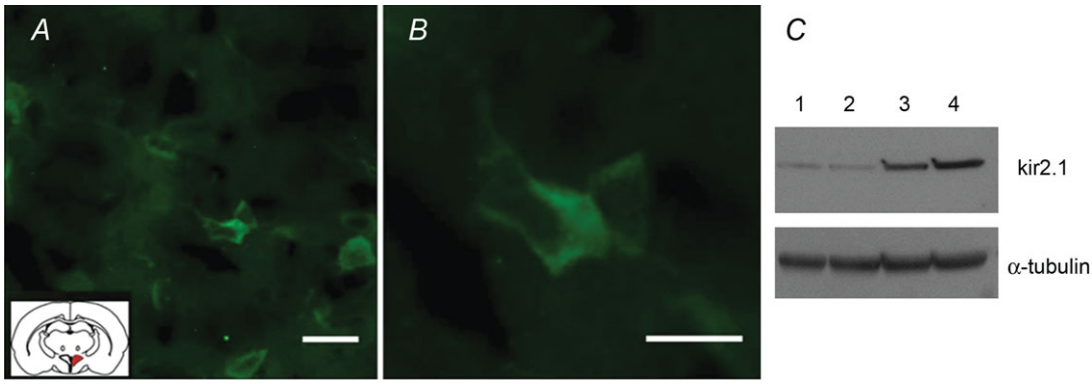

Figure 4. Lentiviral vector-mediated transduction of enhanced green fluorescent protein (eGFP) in the paraventricular nucleus
Confocal microscope images of eGFP-expressing cells in the paraventricular nucleus following injection of lentiviral vector into this site. Scale bar in *A* represents 20 μm and scale bar in *B* represents 10 μm. *C*, Western blot analysis of sham-treated SHRs (lanes 1 and 2) and LVV-hKir2.1 microinjected SHRs (lanes 3 and 4). Results show an overexpression of hKir2.1 in LVV-hKir2.1-microinjected SHRs. α-Tubulin was used as the housekeeping gene.

neurones, in order to evaluate its consequences upon long-term blood pressure regulation in an animal model of hypertension. We overexpressed a human inwardly rectifying potassium channel (hKir2.1) under the control of a synapsin promoter that was neurone specific (Duale *et al.* 2005*a*; Duale *et al.* 2005*b*). Lentivirus was used because its expression has been shown to be sustained within PVN neurones in the long term (Coleman *et al.* 2003). In previous studies, Duale *et al.* (2007) and Howorth *et al.* (2009) showed that hKir2.1 overexpression hyperpolarized the membrane potential of cultured cathecolaminergic PC12 cells by ∼10 mV, which is expected to result in 'electrical silencing' of PVN neurones (Duale *et al.* 2007; Howorth *et al.* 2009). Similar overexpression strategies have been used to reveal that electrical silencing of neurones affected development *in ovo* (Yoon *et al.* 2008), neuronal activity *in vivo* (Okada & Matsuda, 2008) and the ability of neurones to make and maintain connections *in vivo* (Yu *et al.* 2004; Mizuno *et al.* 2007; Hendy, 2010). This virus-mediated approach has the advantage of being site specific and enabling overexpression in adulthood, which avoids the development of putative compensatory mechanisms associated with transgenic animals (Hendy, 2010).

Our results show that LVV-hKir2.1 treatment of the PVN in SHRs lowered SBP by ∼15% (>20 mmHg). This decline of SBP, which was accompanied by a decrease in HR, was statistically confirmed at 30 days after the lentiviral microinjection and persisted until the animals were killed 60 days postinjection. Interestingly, the LF spectra of SBP (indicative of sympathoinhibition) occurred before the fall in SBP (i.e. 20 *versus* 30 days), suggesting a putative association between the changes in both variables. Furthermore, the fall in HF SBP is indicative of reduced respiratory modulation of arterial pressure and could include reduced respiratory–sympathetic coupling, a phenomenon known to raise total peripheral resistance in the SHR (Simms *et al.* 2009). In contrast, changes in diastolic BP were significant only after 50 days, suggesting the involvement of an additional mechanism. This reveals novel insight into the long-term control of arterial pressure in hypertension by the PVN. It also indicates that the system does not adapt. This could be explained by the associated improvement of baroreflex gain and/or a downregulation of peripheral chemoreflex responsiveness to stabilize lower levels of blood pressure, as we observed. We propose that these changes were a result of reduced electrical excitability of PVN premotor sympathetic neurones, but we cannot rule out reduced release of vasopressin and oxytocin. This is consistent with our neuroanatomical Western blot analysis confirming that hKir2.1 protein overexpression was within the PVN region. Interestingly, respiratory rate remained unchanged in all experimental groups, suggesting that there is no tonic excitatory drive from the PVN affecting this variable in hypertensive or normotensive rats. Additionally, we saw no tonic influence from the PVN on the resting arterial pressure level in normotensive rats, which contrasts with a previous acute *in vivo* study (Allen, 2002).

It is well accepted that neurogenic hypertension is accompanied by an impairment of the baroreceptor reflex (Grassi *et al.* 1998). Our data showed that depressing PVN neuronal activity improved baroreflex gain. Previous work from several authors has shown that during the course of an alerting reaction there is a decrease in baroreflex efficacy and a facilitation of the carotid chemoreceptor reflex due to modifications of synaptic integration at the level of the nucleus tractus solitarii; this might include mechanisms involving GABA and angiotensin II release within the nucleus tractus solitarii (Jordan *et al.* 1988; Spyer K, 1990; Silva-Carvalho *et al.* 1995*a*,*b*; Kasparov *et al.* 1998; Kasparov & Paton, 1999; Head & Mayorov, 2001; Rocha *et al.* 2003). Such an angiotensinogenic mechanism seems to be particularly active in pathophysiological conditions such as myocardial ischaemia and hypertension (Rocha *et al.* 2003; Rosário *et al.* 2003; Maximino *et al.* 2006), and its behaviour can be modulated by intervening pharmacologically on AT_1 receptors within the nucleus tractus solitarii (Kasparov *et al.* 1998; Kasparov & Paton, 1999; Rocha *et al.* 2003; Rosário *et al.* 2003). In fact, during myocardial ischaemia, AT_1 blockade reversed the remodelling of baroreceptor and chemoreceptor reflex function in a way similar to that elicited upon the overexpression of hKir2.1 in PVN neuronal cells (Rocha *et al.* 2003; Rosário *et al.* 2003).

The demonstration of a non-dipper blood pressure profile in animal models remains difficult, mainly due to the failure to establish a clear distinction between day and night values. This was confirmed in our study, because through PVN-induced sympathetic manipulations, we were only able to modify BP light–dark values of SHRs which approached those of WKY rats. However, we were unable to modify the day and night profile of BP value variations in both strains. This inability to define a light–dark profile in rats similar to the one set for human subjects may be due to the intermittent behaviour rats, with alternating awake and sleep periods in both the light and the dark phase. It is likely that the only way to define the light and dark phase profiles of rats better would be by monitoring of cerebral activity through EEG, which was outside the scope of the present work.

In conclusion, the present work shows that the intervention on central sympathoexcitatory neurone excitability through the genetic manipulation of expression of K^+ channels is able to alter peripheral blood pressure in the long term. This occurs by remodelling of the sympathetic outflow and restores the imbalance of peripheral reflex mechanisms that maintain cardiovascular homeostasis. Our data, from an

animal model, give insights into the pathophysiological mechanisms involved in the aetiology of neurogenic hypertension and provide novel hypothetical therapeutic interventions at both the central and the peripheral level of the autonomic nervous system to control sympatoexcitation.

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Additional Information

Competing interests

None declared.

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